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REMARKS

Claims 1-5, 54, 56, 58-61, 63-70, 78-79, 82-83 and 104-107 are pending. Claim 54 has been amended to correct a minor clerical error. Support for the amending language may be found in originally filed claim 68. No new matter is added. In view of the following remarks, reconsideration and withdrawal of the rejections is requested.

Claims 1-5, 54, 56, 58-61, 63-70, 78-79, 82-83 and 104-107 have been rejected under 35 U.S.C. 112, first paragraph. The Office Action states that the claims contain subject matter which was not described in the specification in such a way as to convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

On page 2, the Office Action goes on to state that each recites a limitation of uroplakin (UPII) transcriptional regulatory element (TRE) that comprises specific nucleotide sequences as set forth in the Seqlist. The Examiner thus acknowledges that the structure of the claimed sequences is clearly described. On page 3, the Office Action further states a requirement that "desired urothelial cell-specific expression is obtained". Hence, the Examiner further acknowledges that the function of the claimed sequences is also clearly described. Applicants agree with this assessment, and point out that the broadest claims are directed to a TRE comprising a nucleotide sequence selected from the group consisting of "nucleotides 2028 to 2239 of SEQ ID NO:1; nucleotides 1647 to 2239 of SEQ ID NO:1; nucleotides 1223 to 2239 of SEQ ID NO:1; nucleotides 1 to 2239 of SEQ ID NO:1; nucleotides 430 to 2239 of SEQ ID NO:1; nucleotides 2023-2239 of SEQ ID NO:1; nucleotides 3005 to 3592 of SEQ ID NO:2; and 2627 to 3592 of SEQ ID NO:2". Each TRE within the scope of the claims, therefore, has an absolute requirement that one of these eight, defined nucleotide sequences will be present.

The specification provides a number of examples of adenovirus vectors constructed with the claimed TRE fragments. For clarity, Applicants provide herein a detailed listing of adenovirus constructs that are described in the present application; with a lettering system to identify the presented claims UPII TREs.

	Sequence	Construct	Independent Claim	Support for urothelial cell specific activity
A	nucleotides 1 to 2239 of SEQ ID NO:1 (human)	CP657; CV876	4	Figs. 5 and 6
B	nucleotides 430 to 2239 of SEQ ID NO:1 (human)	CV882; CV884	5	Fig. 14 and page 117, lines 22-24 of the specification
C	nucleotides 1223 to 2239 of SEQ ID NO:1 (human)	CP656; CV875; CV832; CV833;	3	Figs. 5 and 6

		CV824; CV825		
D	nucleotides 1647 to 2239 of SEQ ID NO:1 (human)	CP663; CV822; CV823	2	Fig. 5
E	nucleotides 2028 to 2239 of SEQ ID NO:1 (human)	CP662	1	Fig. 5
F	nucleotides 3005 to 3592 of SEQ ID NO:2 (mouse)	CP618; CV826; CV827; CV830; CV831; CV818; CV819	64	Fig. 6
G	nucleotides 2627 to 3592 of SEQ ID NO:2 (mouse)	CP619; CV877; CV828; CV829; CV820; CV821	65	Fig. 6

Name	Vector	Ad Vector	TRE	E1A TRE	TRE
CV808	CP569	pBHG10		3.6 kb mUPII	
CV818	CP622	pBHG10	G	0.6 kb mUPII	
CV819	CP622	pBHGE3	G	0.6 kb mUPII	
CV820	CP623	pBHG10	F	1.0 kb mUPII	
CV821	CP623	pBHGE3	F	1.0 kb mUPII	
CV822	CP664	pBHG10	D	0.6 kb hUPII	
CV823	CP664	pBHGE3	D	0.6 kb mUPII	
CV824	CP665	pBHG10	C	1.0 kb hUPII	
CV825	CP665	pBHGE3	C	1.0 kb hUPII	
CV826	CP667	pBHG10	G	0.6 kb mUPII	C
CV827	CP667	pBHGE3	G	0.6 kb mUPII	C
CV828	CP669	pBHG10	F	1.0 kb mUPII	C
CV829	CP669	pBHGE3	C	1.0 kb hUPII	F
CV830	CP672	pBHG10	C	1.0 kb hUPII	G
CV831	CP672	pBHGE3	C	1.0 kb hUPII	G
CV832	CP673	pBHG10	C	1.0 kb hUPII	F
CV833	CP673	pBHGE3	C	1.0 kb hUPII	F

Figure 5 depicts the expression of a luciferase gene operably linked to a number of plasmids comprising UP TREs. Among these are CP648 (human uroplakin Ia TRE); CP649 (human uroplakin Ia TRE); CP662 (TRE fragment E); CP663 (TRE fragment D); CP656 (TRE fragment C); CP657 (TRE fragment A) and CP620 (SEQ ID NO:2, complete sequence). Applicants note that the

plasmids CP648 and CP649, as stated in Table 1, do not contain a UPII transcriptional regulatory element. A review of the expression of the 4 human uroplakins, UPIa, UPIb; UPII and UPIII is attached herewith to clarify this point (Lobban et al. (1998) AJP 153:1957-1967). Applicants also note that no claims are presently pending which claim the TRE that was used in CP620.

The vectors were tested in the following cell lines: HepG2, a hepatocellular carcinoma; LoVo, a colon carcinoma; PA-1, an ovary teratocarcinoma; SW780, a bladder cell carcinoma; SW1463, a rectal adenocarcinoma; and UM-UC3, a bladder cell carcinoma.

As stated in the specification (page 104, lines 9-14), constructs CP648 and CP649 showed no preferential expression in SW780 cells. However, CP618, CP620, CP662, CP663, CP656 and CP657 showed significant preferential expression in SW780 cells, indicating the presence of at least one urothelial cell-specific TRE in each of these constructs.

Therefore, based on the evidence presented in Figure 5, the UPIa promoters show little activity in any of the lines tested, but the CP662; CP663; CP656; CP657 and CP620 constructs all preferentially expressed in urothelial cells. These constructs, to reiterate, included the fragments A, B, D and E of the human UPII TRE.

Figure 6 also provides evidence of TRE activity, and tissue specificity. CP618; CP619; CP1066; CP656; CP657 and CP620 were assayed. All showed greater activity in SW780 than in other cells tested. . Also, the human TRE fragments A (CP657) and C (CP656) are preferentially expressed.

The murine UPII TRE provides for a biologically relevant effect (shown in Figure 11), where a bladder xenograft tumor is dramatically shrunk when treated with CV808.

The last of the figures in the application, Figure 14, compares the wild-type virus CV802 with CV882 and CV884, both of which utilize the UPII TRE B. It can be seen that the replication of CV882 in permissive cells (293, RT-4 and SW780) is comparable to wild-type, while it is substantially reduced in non-permissive cells (G361, LNCap, HBL-100, MKN1, PA-1 and primary cells).

Applicants respectfully submit that a set of clearly defined transcriptional regulatory elements are set forth in the specification and the claims. The elements are utilized in a large number of constructs, and varying configurations (see the above table). The data demonstrate that the TREs provide for expression of adenovirus genes essential for replication, as set forth in Claims 54, 56, 58-61, 63-70, 78-79, 82-83 and 104-107; and further are selectively expressed in bladder cells, as set forth in Claims 1-5.

The Office Action states that “a reasonably broad interpretation of the words ‘uroplakin II TRE’ in light of these teachings encompasses additional transcriptional regulatory elements (TREs) that are associated with the human and murine uroplakin II genes that respond under different conditions in urothelial cells (e.g. at different times during urothelial cell development) that are not present within SEQ ID NOs:1 or 2. The claims also reasonably encompass synthetic TREs that comprise additional elements derived from homologs of UPII obtained from other sources that are not identical to those described in the specification.”

Applicants see no reason why the claimed subject matter should not encompass constructs where the provided – specifically defined and effective – TREs are combined with other regulatory elements, given that the claims recite structure and function clearly described in the specification. There is no question that Applicants have fulfilled the written description of the sequences set forth in the present claims. There is no ambiguity about what is meant by, for example, nucleotides 1647 to 2239 of SEQ ID NO:1. Nor is there any question that enhancers and promoters are widely used in the art, and that such use is enabled by well known assays and methods; including methods such as those provided in the examples of the present application.

It is well-known in the art that enhancers and promoters may be combined. Applicants have provided ample examples of the active fragments in SEQ ID NO1 and SEQ ID NO:2, and have shown the effectiveness of these fragments in cells and in animals. Whether a promoter is obtained from a virus, a synthetic promoter bought from a research supply house, or from the UPII sequence of another species is immaterial.

The Office Action states that “the rejected claims encompass embodiments where the claimed enhancer only comprises portions of the largest human and murine UPII TRE elements described in the instant specification (i.e. the 2.240 kb human UPII TRE described by SEQ ID NO:1 and the 3.592 murine UPII TRE described by SEQ ID NO:2). Because the rejected claims are drawn towards a UPII TRE comprising only portions of the sequences described in SEQ ID NO:1 or SEQ ID NO:2, the rejected claims encompass a very large number of potential TRE sequences.”

Applicants submit that the claims are directed to very specific and carefully described portions of these sequences, which portions are shown to be active enhancers. The statement concerning “a very large number of potential TRE sequences” is not understood. The claims sequences are fully described, and their use is fully enabled. The number of possible nucleotide combinations that could be ligated to the ends does not detract from Applicants compliance with the requirements for written description.

The Office Action states that “the specification teaches only two sequences from different sources that considered to be TRE sequences associated with Uroplakin II genes”. Applicants do not dispute this statement, but point out that, in fact, all of the claims specifically require one of the provided sequences to be present.

The Office Action states that “construct CP619, which comprises an intermediate fragment of 1.0 kb obtained from the human UPII genes showed little expression”. Applicants respectfully draw the Examiner’s attention to Figure 6. While this fragment may have low expression, it is, in fact, a positive enhancer element and it is preferentially expressed in bladder cells. Applicants have met their burden through the disclosure of the sequence and of the biological activity.

The Office Action states that “the specification does not provide a reliable basis for one to envision those embodiments comprising a specific subsequence of SEQ ID NO:1 or SEQ ID NOL2 in addition to the enhancer/promoter elements such that the synthetic construct will retain TRE activity.”

Applicants respectfully submit that the burden for written description has been met by explicitly spelling out the sequences of the TREs to be used with the constructs of the invention; by the demonstrated use of such sequences in dozens of different combinations; and by the demonstrated biological activity of the sequences. These many teachings, in combination with the extensive guidelines provided by the specification and by the wide knowledge in the art with respect to the use of promoter and enhancer elements, more than provides what the law requires.

Finally, the Office Action states that “it is not unreasonable to limit the invention to that which has been described.” Applicants respectfully submit that the presently claimed invention is fully described by the specification. In view of the above remarks, withdrawal of the rejection under 35 U.S.C. 112, first paragraph, is respectfully requested.

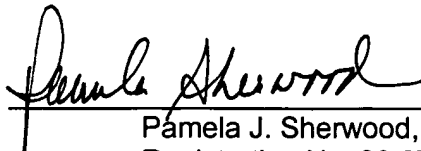
CONCLUSION

Applicants submit that all of the claims are now in condition for allowance, which action is requested. If the Examiner finds that a Telephone Conference would expedite the prosecution of this application, she is invited to telephone the undersigned at the number provided.

The Commissioner is hereby authorized to charge any other fees under 37 C.F.R. §§ 1.16 and 1.17 which may be required by this paper, or to credit any overpayment, to Deposit Account No. 50-0815, order number CELL-016.

Respectfully submitted,

Date: Feb. 20, ²⁰⁰⁴~~2003~~

By: 
Pamela J. Sherwood, Ph.D.
Registration No. 36,677

BOZICEVIC, FIELD & FRANCIS LLP
200 Middlefield Road, Suite 200
Menlo Park, CA 94025
Telephone: (650) 327-3400
Facsimile: (650) 327-3231

Uroplakin Gene Expression by Normal and Neoplastic Human Urothelium

E. Dawn Lobban,* Barbara A. Smith,*
Geoffrey D. Hall,* Patricia Harnden,[†]
Paul Roberts,[‡] Peter J. Selby,*
Ludwik K. Trejdosiewicz,* and Jennifer Southgate*

From the ICRF Cancer Medicine Research Unit* and
Departments of Pathology[†] and Cytogenetics,[‡] St. James's
University Hospital, Leeds, United Kingdom

cDNA sequences for human uroplakins UPIa, UPIb, UPII, and UPIII were cloned and used to investigate uroplakin transcription by normal and neoplastic urothelial cells. Normal urothelium expressed mRNA for all four uroplakins, although UPIII could be detected only by ribonuclease protection assay. By *in situ* hybridization, UPIa and UPII were confined to superficial cells and UPIb was also expressed by intermediate cells. Cultured normal human urothelial cells showed a proliferative basal/intermediate cell phenotype and constitutive expression of UPIb only. Uroplakin expression by transitional cell carcinoma cell lines was related to their differentiated phenotype *in vitro*. RT4 cells expressed all uroplakins, VM-CUB-3 expressed three uroplakins, RT112 and HT1376 cells expressed only UPIb in high abundance, and COLO232, KK47, and EJ cells had no detectable expression. These results correlated with patterns of uroplakin expression in tumors. UPIa and UPII were detected superficially only in well differentiated transitional cell carcinoma papillae. UPIb was positive in seven of nine and overexpressed in five of nine non-invasive transitional cell carcinomas and was also present in four of eight invasive transitional cell carcinomas. Lymph node metastases retained the same pattern of UPIb expression as the primary tumor. Unlike the three differentiation-regulated uroplakins, UPIb may have an alternative role in urothelial cell/tissue processes. (Am J Pathol 1998; 153:1957-1967)

Urothelium is a stratified epithelium in which basal, intermediate, and superficial cell zones can be distinguished on the basis of morphology and differential antigen expression. The superficial or umbrella cell is characterized by a unique asymmetric unit membrane, which is recognized as an unequivocal ultrastructural marker of terminal urothelial cytodifferentiation.¹ The asymmetric unit membrane is present on the apical plasma membrane surface as thickened membrane plaques interconnected by re-

gions of normal trilaminar membrane (interplaque or hinge regions). Antisera reacting, respectively, with interplaque and plaque regions of the specialized apical membrane have been developed as markers of urothelium-specific cytodifferentiation.^{2,3}

Characterization of the asymmetric unit membrane from bovine urothelium has led to the identification of specialized integral membrane proteins known collectively as the uroplakins.⁴ Four highly conserved uroplakin (UP) proteins have been characterized. The 27,000 M_r UPIa and the 28,000 M_r UPIb proteins belong to the tetraspan protein family⁵ and in bovine urothelium are thought to interact, respectively, with the genetically unrelated 15,000 M_r UPII and 47,000 M_r UPIII proteins.^{6,7} On the basis of their urothelial specificity and differentiation-restricted expression, the uroplakins have been described as urothelial differentiation-related membrane proteins and proposed as markers of advanced urothelial cytodifferentiation.³

Although well characterized in the bovine system, less is known regarding the expression of uroplakins in normal and neoplastic human urothelia, where studies have been hampered by a lack of monospecific reagents recognizing individual uroplakin proteins *in situ*. Expression of UPIII in normal and neoplastic human urothelium has been examined by comparing the immunolocalization patterns of two rabbit heteroantisera raised against isolated bovine asymmetric unit membranes, which were considered to co-localize where UPIII was expressed.⁸ By this criterion, UPIII was restricted to the apical membrane of superficial umbrella cells of normal urothelium and was retained by 88% of papillary noninvasive transitional cell carcinomas (TCCs), 53% of invasive TCCs, and 66% of metastatic TCCs, suggesting a possible diagnostic application.⁸ Similar results were attained using an anti-UPII peptide antiserum.^{8,9}

Normal human urothelial (NHU) cells can be maintained in a highly proliferative state in serum-free culture in a low-calcium medium, where they express a basal/intermediate cell phenotype.¹⁰ Stratification can be induced by increasing the exogenous calcium concentration but is not accompanied by expression of asymmetric unit-membrane-associated antigens. This implies that the

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Address reprint requests to Dr. J. Southgate, ICRF Cancer Medicine Research Unit, St. James's University Hospital, Leeds LS9 7TF, UK. E-mail j.southgate@leeds.ac.uk.

Table 1. *In Situ* Hybridization of Uroplakins on Normal and Neoplastic Urothelium

Stage/Grade	Sex	Age (years)	UPIa	UPIb	UPII
Normal					
	F	ND	S+	S+	S+
	F	ND	S+	S+	S+
TCC					
pTa/G1	F	74	S+	S+	S+
pTa/G1	M	51	S+	D++	S+
pTa/G2	M	62	-	-	-
pTa/G2	M	76	S+	-	S+
pTa/G2	M	79	S+	D++	S+
pTa/G2	F	82	-	D++	S+
pTa/G2-3	M	67	S+	S+	S+
pTa/G3	M	94	-	D++	-
pTa/G3	M	83	-	D++	S+

F, female; M, male; ND, no data; D, diffuse; S, superficial; -, negative.

processes that regulate stratification and differentiation in urothelium are independent.¹⁰ The molecular mechanisms regulating cytodifferentiation in human urothelial cells are poorly understood but are critical to normal urinary tissue function, as the asymmetric unit membrane is regarded as important to both the barrier and accommodating properties of the urothelium. Loss of differentiation is associated with poor prognosis in TCC,¹¹ whereas retention of differentiation, as evidenced by the restricted superficial expression of cytokeratin CK20, may have a role in predicting nonrecurrence in noninvasive papillary TCC.¹² To investigate the expression of differentiation-associated genes in human urothelium, we have studied expression of the individual uroplakin transcripts in normal and neoplastic urothelial cells.

Materials and Methods

Tissues

Surgical specimens of bladder, ureter, and renal pelvis were obtained from patients with no history of urothelial dysplasia or malignancy. Tissues were collected in Transport medium consisting of Hanks' balanced salt solution (HBSS) containing 10 mmol/L Hepes, pH 7.6, and 20 kIU of aprotinin (Trasylol, Bayer, Newbury, UK), as described.¹⁰ Representative pieces from each tissue sample were processed into paraffin wax for immunohistology and *in situ* hybridization. The remaining sample was cut into approximately 1-cm² pieces, placed into Transport medium (Ca²⁺ and Mg²⁺ free) containing 0.1% (w/v) EDTA, and incubated at 4°C overnight to release the urothelium. The isolated urothelium was either used to initiate normal human urothelial (NHU) cell lines or used to extract RNA (below).

Paraffin-wax-embedded specimens of nine noninvasive TCCs and eight invasive TCCs, five with nodal metastases, were obtained from the General Infirmary, Leeds, as detailed in Tables 1 and 2. Paraffin-wax-embedded specimens of normal human urinary tract, kidney, lung, and duodenum were included as specificity controls for *in situ* hybridization studies.

Table 2. UPIb and CK20 Expression on Invasive and Metastatic TCC

Stage/Grade	Sex	Age (years)	UPIb <i>in situ</i> hybridization	CK20 immunohistology
pT1/G2-3	F	75	D++	ND
pT2/G2-3	M	62	-	+
pT2/G3	F	92	S+	+
pT2/G3	M	62	-	+
LNM			-	+
pT3b/G3	F	51	D++	+
LNM			D++	+
pT3b/G3	F	64	-	-
LNM			-	-
pT4	M	53	-	-
LNM			-	-
pT4	M	71	D++	+
LNM			D++	-

F, female; M, male; D, diffuse; S, superficial; ND, no data; -, negative; LNM, lymph node metastasis.

Cell Culture

NHU cell lines were established as previously described¹⁰ and maintained in keratinocyte serum-free medium (KSFM) containing bovine pituitary extract and epidermal growth factor at the manufacturer's recommended concentrations (Gibco BRL, Paisley, UK) and 30 ng/ml cholera toxin (Sigma Chemical Co., Poole, UK).

Seven established human TCC cell lines were used, comprising RT4, RT112, HT1376, COLO232, KK47, VM-CUB-3, and EJ. These cell lines are well characterized and show a range of phenotypes reflecting the urothelial origin and the different grades of cancer from which the lines were derived.¹³⁻¹⁶ The cell lines were maintained in a 1:1 mixture of RPMI 1640 and Dulbecco's minimal essential medium with 5% fetal bovine serum, as described previously.¹³ The colonic adenocarcinoma cell line DLD-1 was obtained from the American Type Culture Collection and included as a non-urothelial control in the ribonuclease protection assays (RPAs; see below). All cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air and passaged at near confluence by incubation in 0.1% (w/v) EDTA in PBS for 5 minutes followed by detachment in 0.25% (w/v) trypsin in 0.02% (w/v) EDTA.

RNA Extraction

Detached urothelial cell sheets or cultured cells were solubilized in Ultraspec (Biogenesis, Poole, UK). Isolation of RNA by chloroform extraction and isopropanol precipitation was performed according to the manufacturer's instructions (Biogenesis). The concentration of total RNA was estimated from the A₂₆₀ reading, and quality was assessed on agarose formaldehyde gels.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

To prepare cDNA, 1 µg of RNA and 100 ng of oligo dT (Sigma) in a final volume of 20 µl were heated to 65°C for

5 minutes and cooled on ice; 10 μ l was removed into 10 μ l of reaction buffer so that final concentrations in 20 μ l were 10 mmol/L Tris, pH 8.3, 50 mmol/L KCl, 8 mmol/L $MgCl_2$, 1 mmol/L dNTPs, and either 5 U of MMLV reverse transcriptase (Pharmacia Biotech, St. Albans, UK) (RT^+) or the equivalent volume of water (RT^- control). The reaction mix was incubated at 37°C for 60 minutes before denaturing at 95°C for 10 minutes. In some experiments, RNA was treated with DNase before cDNA synthesis using a Message Clean Kit (Gene Hunter, supplied by Bio/Gene, Bolnhurst, UK).

PCR was performed on samples of the RT^+ and RT^- reactions by removing 10- μ l aliquots into tubes containing PCR components so that the final concentrations in a 50- μ l reaction volume were 10 mmol/L Tris, pH 8.3, 50 mmol/L KCl, 0.2 mmol/L dNTPs, and 1.6 mmol/L $MgCl_2$. Each tube also contained 50 pmol each of forward and reverse primers (below) and 1.25 U of *Taq* polymerase (Perkin Elmer Cetus, supplied by Applied Biosystems, Warrington, UK). After an initial denaturation step at 95°C for 5 minutes, PCR was carried out for 30 cycles and products were analyzed by electrophoresis on 2% agarose gels. For cloning, bands were excised and purified on QIAquick gel extraction columns (Qiagen, Crawley, UK).

Cloning Human Uroplakin Sequences

Partial cDNA sequences for UPIa, UPIb, UPII, UPIII, and GAPDH were amplified from human urothelial cDNA by PCR and cloned into vectors as described below.

UPIa

The cDNA sequence for UPIa was assembled from the genomic sequence on the GenBank database (AC002115), and primers were chosen from the third and fifth exons. Using forward primer GCTCATCGTCTA-CATCTCGA and reverse primer CTGACGTGAAGT-TCACCCAG, the resulting PCR fragment was TA-cloned into the pGEMT-Easy vector using the manufacturer's protocol (Promega, Southampton, UK), and the sequence and insert orientation were determined by ABI PRISM dye terminator cycle sequencing (Perkin Elmer) and analysis on an ABI 377 sequencer (Applied Biosystems).

UPIb

The GenBank cDNA sequences for bovine UP1b (Z29378) and for the related TI1 gene from mink lung (M64428) were aligned using PileUp from the GCG program suite. Forward TTCCAGGGCCTGCTGATTTT and reverse CATGGTACCCAGGAGAACCC primers chosen from regions of sequence identity within the coding region were used to amplify a cDNA product that was cloned into pGEM-T Easy and sequenced as above.

UPII

The cDNA sequences for murine (UO8030) and bovine (L20633) UPII were obtained from the GenBank databases and aligned using PileUp. Two forward and two reverse complement sequences of 20 bp each were selected as PCR primers from regions of identity and used in various combinations to perform RT-PCR on human urothelial RNA under low stringency (45°C annealing). All primer combinations produced several bands. However, with forward primer GATCCTGATTCTGCTG-GCTG and reverse primer ATGTAGTATTGGTTCCTGG, a product was amplified at the expected size of 264 bp. The 264-bp product was inserted into the *Sma*I site of the pGEM3Z vector (Promega) by TA cloning, and the sequence and insert orientation were determined. The sequence was shown to have good sequence homology with part of the coding region of both bovine and mouse UPII cDNA. A ^{32}P -labeled riboprobe was produced from the clone and used in *in situ* hybridization studies and RPAs and to screen a human urothelial cDNA library (below).

UPIII

The partial sequence for human UPIII was obtained from the GenBank data base (AA513520), and forward GGAGGCATGATCGTCATCAC and reverse TCACG-GACGTGTAGGAAGAC primers were chosen to give a PCR product of 193 bp. RT-PCR was carried out on human urothelial RNA, and the product was cloned into the pGEM-T Easy vector. The insert sequence and orientation were determined.

GAPDH

A 152-bp portion of the coding region (position 511 to 655) of human GAPDH (M33197) was cloned into the pGEM-T Easy vector. Radiolabeled riboprobe produced from the T7 promoter site was used as the internal control in RPAs (below).

In Situ Hybridization

Specific uroplakin mRNA transcripts were localized on paraffin-wax-embedded sections using [^{35}S]UTP-labeled riboprobes.¹⁷ A β -actin riboprobe was used to determine RNA transcript integrity within the tissue.¹⁸ To produce labeled riboprobes, 500 ng of linearized template was transcribed from T7 or SP6 as appropriate using a riboprobe *in vitro* transcription kit (Promega) incorporating [^{35}S]UTP (Amersham Life Sciences, Little Chalfont, UK). The template was destroyed by incubating with 0.5 U of DNase (Promega) for 15 minutes at 37°C, and the reaction was diluted in 10 mmol/L EDTA containing 1.5 μ g of rRNA as carrier and purified on a Chromaspin-30 DEPC column (Clontech, supplied by Cambridge Biosciences, Cambridge, UK).

Five-micron paraffin sections were collected onto clean, baked, 3-aminopropyltriethoxysilane-coated slides.¹⁹ Slides

were dried overnight at 40°C, dewaxed in xylene containing 0.1% (v/v) DEPC for 8 minutes, and rehydrated through graded DEPC-treated alcohols to PBS. Sections were digested with 20 µg/ml proteinase K at 37°C for 10 minutes, stopped in 0.2% (w/v) glycine, washed in PBS, post-fixed for 20 minutes in 4% (w/v) paraformaldehyde, and washed in PBS. The tissue was acetylated for 10 minutes in 0.1 mol/L triethanolamine and 0.25% (v/v) acetic anhydride, washed in PBS, dehydrated through DEPC-treated graded alcohols, and air dried.

Labeled probes were diluted in a ratio of 1:5 with hybridization buffer (0.3 mol/L NaCl, 10 mmol/L Na₂HPO₄, 10 mmol/L Tris/HCl, pH 6.8, 5 mmol/L EDTA, 0.02% (v/v) Denhardt's solution, 50% (v/v) formamide, 200 µg/ml rRNA, 10% (w/v) dextran sulfate, and 10 mmol/L dithiothreitol), heated to 80°C for 1 minute, and applied to give a final activity of 2×10^6 cpm/slide. After overnight incubation at 55°C, slides were washed extensively at 55°C in 50% formamide buffer and then in TNE buffer (10 mmol/L Tris/HCl, pH 7.4, containing 0.5 mol/L NaCl and 1 mmol/L EDTA). Sections were incubated in 100 µg/ml RNase A in TNE buffer at 37°C for 1 hour to digest unbound probe, washed twice in 2X SSC at 65°C for 30 minutes, 0.5X SSC at 65°C for 30 minutes, and at high stringency in 0.1X SSC at 65°C for 30 minutes before dehydrating through graded alcohols containing 0.3 mol/L ammonium acetate. Slides were air dried overnight, coated with K5 autoradiographic emulsion (Ilford, Knutsford, UK), and left at 4°C for 10 to 20 days before development. Sections were counterstained with Mayer's hematoxylin, air dried, and mounted in Eukitt (Merck, Poole, UK).

Ribonuclease Protection Assay

Antisense RNA transcripts of high specific activity were generated under the control of the T7 or SP6 promoter, as appropriate. Approximately 100 ng of gel-purified linearized template was transcribed using a riboprobe *in vitro* transcription kit (Promega) incorporating [³²P]CTP (Amersham International, Little Chalfont, UK). The DNA template was destroyed by a 15-minute incubation in the presence of 1.5 U of RNase-free DNase (Promega), and 10 µg of yeast tRNA was added as a carrier for the riboprobes, which were either phenol:chloroform extracted and ethanol precipitated or purified on Chromaspin 30-DEPC columns (Clontech).

Specific RNA transcripts were detected using the RPAII RPA kit (Ambion, Austin, TX). A total of 2 fmol of labeled probe was mixed with 5 µg of total RNA (sample or yeast control). Hybridization and digestion were performed according to the manufacturer's instructions (Ambion). After precipitation and electrophoresis on 5% denaturing polyacrylamide gels (Sequagel from Flowgen Instruments, Lichfield, UK), protected probe fragments were visualized by autoradiography.

Human Urothelial cDNA Library

For construction of the cDNA library, total RNA was extracted from isolated normal human urothelium pooled

from five independent donors. mRNA was extracted on polydT-conjugated Dynabeads (DynaL (UK), Bromborough, UK), used according to the manufacturer's instructions. The cDNA library was constructed in pCDM8.²⁰

Approximately 20,000 colonies were plated on Hybond N+ (Amersham) on two 15-cm plates of L agar containing 10 µg/ml each of ampicillin and tetracycline. Replica plating, colony fixation, and hybridization with the ³²P-labeled human UPII riboprobe (above) were all carried out according to Amersham protocols.

Immunohistochemistry

Immunolabeling with monoclonal anti-CK20 was carried out as previously described.^{12,19} Antibody labeling of paraffin-embedded tissue sections was visualized using an indirect streptavidin ABC immunoperoxidase technique (Dako, Ely, UK) after antigen retrieval by digestion for 10 minutes at 37°C in 0.1% trypsin in 0.1% CaCl₂, pH 7.8, and blocking of endogenous avidin binding sites in the urothelium using a kit from Vector Laboratories (Bretton, UK).

Cytogenetic Analysis

Cultures of bladder cancer cell lines in exponential growth were exposed to 0.2 µg/ml colcemid (Sigma) in growth medium for 17 hours. Cells were removed from the substrate with a solution of 0.25% (w/v) trypsin and 0.02% (w/v) EDTA in PBS for 5 minutes and incubated for 15 minutes in 0.075 mol/L KCl before washing three times in Carnoy's fixative. Preparations were analyzed by GTL banding, and three to six metaphases were examined for each cell line. Specific analysis was confined to chromosome 3q status.

Results

Cloning and Analysis of Human Uroplakin cDNA Sequences

UPIa

A 221-bp cDNA fragment was amplified from human urothelial mRNA using primers designed from the coding sequence of the human genomic UPIa sequence. The cloned sequence showed absolute identity with the predicted region of the available human UPIa genomic sequence.

UPIb

A 726-bp sequence was cloned from human urothelial mRNA using consensus primers from within the bovine UPIb and mink T11 coding regions. A BLAST analysis of the nucleotide sequence revealed 90% identity to bovine UPIb and 91% identity to mink T11 (Figure 1). No significant homology was found with any other database sequence. Because UPIa and UPIb are both members of

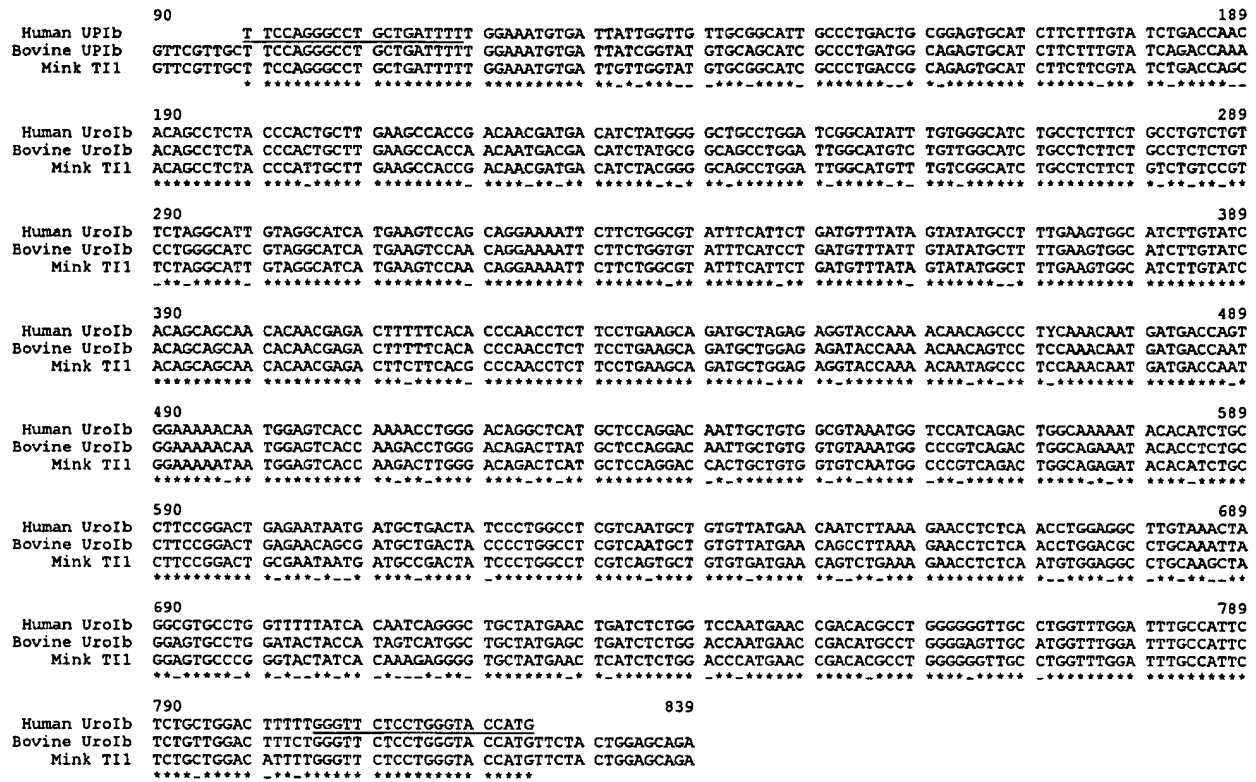


Figure 1. DNA sequence alignment of the partial coding sequence for human UPIb with bovine UPIb and mink TI1 cDNA sequences. The partial human UPIb clone commences at nucleotide position 99 and terminates at position 825 of the full bovine UPIb cDNA sequence. The primers used for PCR amplification of the human sequence, which were designed from consensus regions of the bovine UPIb and mink TI1 genes, are underlined. Identical bases are marked with asterisks.

the tetraspan family, an explicit nucleotide comparison was performed and revealed 45% identity, but in no region was consensus maintained sequentially over more than nine bases. At the amino acid level, the identities were 92% to bovine UPIb, 94% to mink TI1, and 40% to human UPIa. On the basis of the comparison scores at nucleotide and amino acid levels, it was considered that the 726-bp clone was human UPIb. The partial human UPIb cDNA sequence was submitted to the EMBL database (accession number AF082888).

UPII

In first-round screening of the human urothelial cDNA library, 20 colonies hybridized strongly with the UPII riboprobe, indicating a frequency of approximately 0.1% in the library. Analysis of three clones revealed the cloned cDNA to be 920 bp (Figure 2), which coded for a product of 184 amino acids. BLAST analysis of the nucleotide sequence revealed 85% identity with murine UPII and 88% identity with bovine UPII. In addition, the translated human sequence showed 87% and 89% identity with the amino acid sequences for murine and bovine UPII, respectively. The full human UPII cDNA sequence was submitted to the EMBL database (accession number Y13645).

UPIII

The nucleotide sequence of the 193-bp cDNA clone showed absolute identity to the assigned region of the available human UPIII cDNA sequence (accession number AA513520).

Localization of Uroplakin Transcripts in Normal Tissues

In situ labeling with the β -actin probe demonstrated preservation of mRNA transcripts within paraffin-wax-embedded specimens of normal human kidney, duodenum, lung, and ureter (Figure 3). Hybridization of ureter with probes to UPIa and UPII demonstrated highly restricted patterns of expression, with transcripts localized exclusively to the superficial cells of urothelium (Figure 3). Although also differentiation restricted, UPIb mRNA expression was more extensive, with transcripts detected in both superficial and upper intermediate cells (Figure 3). No hybridization was found with the UPIII riboprobe on any specimen of normal ureter ($n = 3$). None of the uroplakin probes showed any specific hybridization to any of the non-urothelial tissues studied (kidney, lung, and duodenum).

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001  GAAAGCCTGCCAGCACCTATTCCACCTCCAGCCGACGATGGCACCCTGCTGCCATCC
                                M A P L L P I R
061  GGACCTTGCCTTGATCTGATTCTGCTGGCTCTGCTGCCAGGGCTGCAGACTTCA
    T L P L I L I L L A L L S P G A A D F N
121  ACATCTCAAGCCTCTCTGGTCTGCTGTCCCGGGCTAACGGAGAGCCTGCTGGTGGCT
    I S S L S G L L S P A L T E S L L V A L
181  TCCCCCTGTACCTTCACAGGAGCAATGCCACACTGATGGTCCGGAGAGCAATGACA
    P C H L T G G N A T L M V R R A N D S
241  GCAAGTGGTGACCTCCAGCTTTGTGGTGCCTCCGTGCCGTGGGCGCAGGGAAGTGGTGA
    K V V T S S F V V P P C R G R R E L V S
301  GTGTGGTGGACAGTGGTGGCTTCACAGTCACTCGGCTCAGTGCATACCAAGTGACAA
    V V D S G A G F T V T R L S A Y Q V T N
361  ACCTCGTGGCAGGACCAATTCTACATTTCTACCTAGTGAAGAAGGGGACAGCACTG
    L V P G T K F Y I S Y L V K K G T A T E
421  AGTCCAGCAGAGATCCCAATGTCCACACTCCCTCGAAGGAACATGGAATCCATTGGGC
    S S R E I P M S T L P R R N M E S I G L
481  TGGTATGGCCCGACAGGGGGCATGGTGGTCACTACGGTGGTCTCTGTGCCATGT
    G M A R T G G M V V I T V L L S V A M F
541  TCCTGCTGGTGGCTGGCTTCATCATTGCCCTGGCACTGGGCTCCCGAAGTAAGAGGTC
    L L V L G F I I A L A L G S R K *
601  TGCCCCGAGCAGCAGCTTCTCCAGGAAGCCAGGGCACCATCCAGTCCCCAGCCACCT
661  GCTCCAGGCCCCAGGCTGTGGCTCCCTTGGTGGCTCGCTCCTCCTCGGCTCGCT
721  CTCCCTAGAGGCTCTCTCCCTCTGTCCCTCTCCTTGGCCCAAGTCCCTCAGCTTCCA
781  ACACTCCATTATCTCTCAGCCCACTCTGTCAAGTGAATTTCTCCCATTTACCA
841  CTTTAAACACCCCATACCAATTCCCCATCTTCACTGAATTAAGTCCCTATAATAAAG
901  GCTGAGGCTGCATCTGCCCAAAAAA
  
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Figure 2. The DNA and deduced amino acid sequence of human UPII cDNA. The asterisk indicates the stop codon TAA at position 591. The presumed polyadenylation signal AATAAA is in bold. The 264-bp partial cDNA sequence used for riboprobes is underlined.

Uroplakin Expression in Normal Tissues by RPA

Although GAPDH was included to assess RNA quantities, it was found that, compared with cells or other tissues, freshly isolated urothelium expressed low amounts of GAPDH relative to the total amount of RNA included in the RPA reaction. This probably reflects the quiescent nature of urothelium *in vivo*. The uroplakin riboprobes did not hybridize to RNA from yeast or from the DLD-1 colonic carcinoma cell line, consistent with the urothelial specificity of uroplakin gene expression.

All four uroplakin transcripts were detected in mRNA extracted from human ureters. After specific hybridization and RNase digestion, the uroplakin riboprobes produced protected bands of the expected sizes of 221 bp (UPIa), 726 bp (UPIb), 264 bp (UPII), and 193 bp (UPIII). A qualitative assessment of the amount of transcript was made by comparing relative band intensities. The most highly expressed transcripts were UPIa and UPII, which were comparable to the signal for GAPDH. UPIII was the least abundant transcript.

Uroplakin Expression by NHU and TCC Cell Lines

Assessment of six independent NHU cell lines grown as monolayer cultures showed that all cell lines expressed UPIb. Weak expression of UPIa was found in one of five and UPII in one of eight NHU cell lines. UPIII was not detected in any of the four NHU cell lines tested. Unlike normal urothelium *in vivo*, a strong signal representing the presence of human GAPDH transcripts was detected in all NHU cultures.

In the TCC cell lines, uroplakin mRNA expression correlated with a differentiated phenotype. RT4 cells, associated with the most differentiated phenotype *in vitro*, expressed all four uroplakin transcripts; UPIb and UPII showed high level expression, whereas UPIa and UPIII were expressed at

lower abundance. The moderately differentiated RT112 and HT1376 cell lines expressed high levels of the UPIb mRNA but did not transcribe any of the other uroplakin genes. The three most anaplastic cell lines (COLO232, KK47, and EJ) contained no detectable uroplakin transcripts. The VM-CUB-3 cell line expressed transcripts for UPIa, UPIb, and UPII, but showed no expression of UPIII. These results are summarized in Table 3.

In Situ Hybridization on Tumors

In situ labeling for β -actin demonstrated preservation of RNA within tumor sections, with occasional negative areas reflecting regions of tissue necrosis. The β -actin was more strongly expressed in the tumors compared with normal tissue samples.

The nine non-invasive tumors showed limited expression of mRNA for UPIa and UPII. Both transcripts were detected along the luminal edges of well differentiated papillary fronds and dysplastic flat mucosa, localizing to the superficially positioned cells, as seen in the normal tissue (Figure 4a). Poorly differentiated areas of tumors were negative. Although UPIb was negative on two non-invasive TCCs and showed a normal superficial pattern on two of nine pTa tumors, the other five specimens were all found to contain high levels of UPIb transcripts with no differentiation-associated restriction, being present throughout the urothelium of papillary fronds (Figure 4b). These results are summarized in Table 1.

The dysregulation of UPIb mRNA expression after neoplastic transformation was further investigated in eight primary invasive tumor samples and their lymph node metastases (Table 2). Although four of the tumors had lost expression of UPIb mRNA in both the primary and lymph node metastasis, the other four primary tumors showed strong expression of UPIb throughout the urothelium (Figure 4c). The extensive expression of UPIb by these tumors was found to be retained after their metastasis to local lymph nodes (Figure 4d).

The expression of CK20 in invasive TCC did not correlate fully with UPIb expression. CK20 expression was retained in five of seven invasive tumors whereas UPIb was positive in only three specimens from the same group. However, whereas UPIb was coordinately expressed on all five primary and metastatic tumor pairs, CK20 showed a differential loss of expression from the metastasis of one case.

Cytogenetic Analysis of TCC Cell Lines

Cytogenetic analysis of the cell lines revealed karyotypes ranging from near diploid to near pentaploid with complex rearrangements of some chromosomes and two to six copies of chromosome 3. In RT4 cells, all copies of chromosome 3 appeared karyotypically normal. The RT112 and KK47 cell lines had 3q abnormalities affecting the 3q13.3–3q21 region of interest. Specifically, RT112 cells had two copies of chromosome 3, one of which had a 3q rearrangement (del(3)(q12q21)), and KK47 had

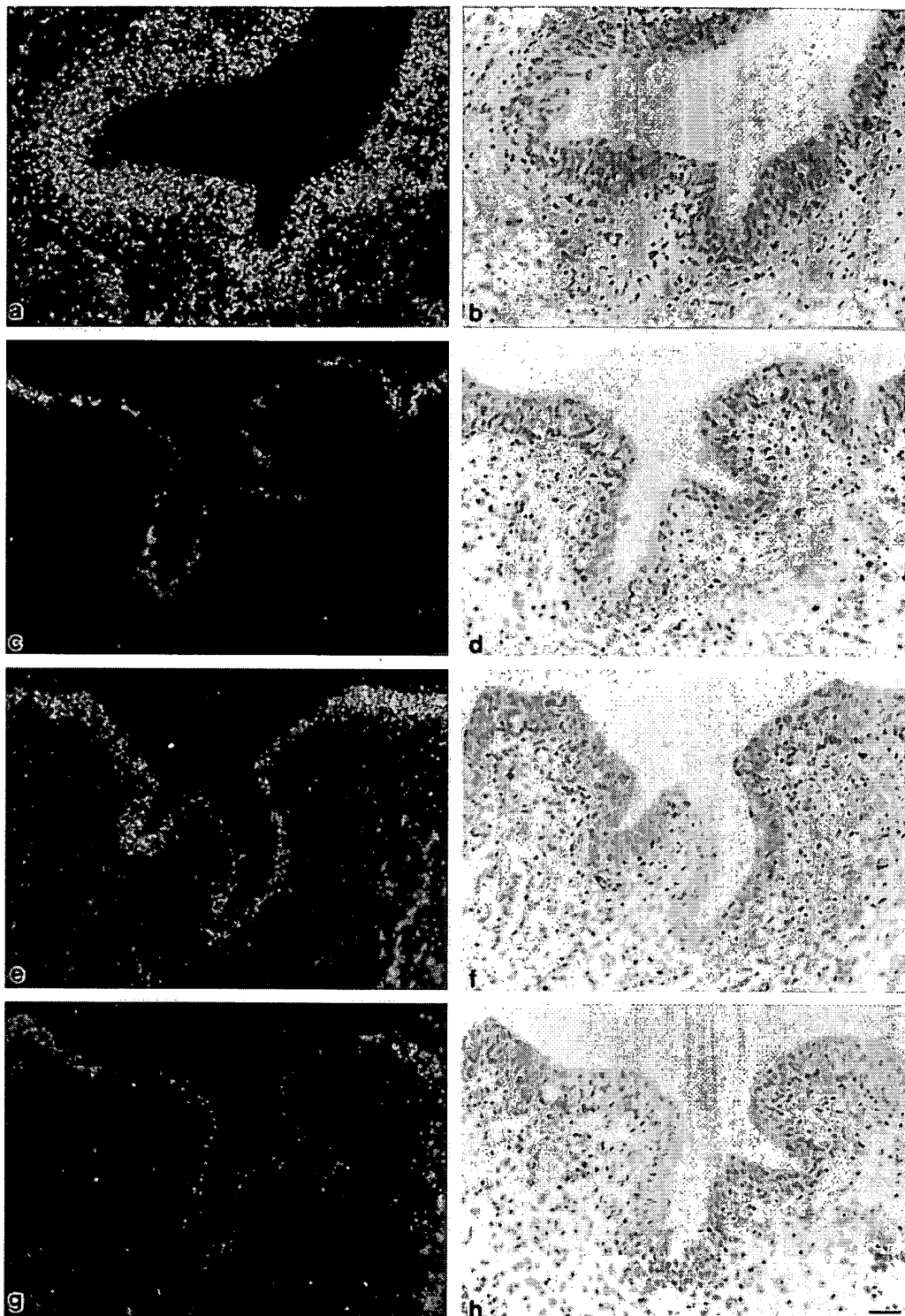


Figure 3. *In situ* hybridization on normal sections of human ureter using ^{35}S -labeled riboprobes detected by autoradiography and visualized by epi-polarized illumination (a, c, e, and g) or transmitted light (b, d, f, and h). Riboprobes were specific for β -actin (a and b), UPIa (c and d), UPIb (e and f), and UPII (g and h). Localization of UPIa and UPII transcripts was confined to the superficial layer of the urothelium, whereas UPIb was also located in cells of the intermediate zone. Scale bar, 50 μm .

three copies of chromosome 3 including one with a 3q rearrangement (del(3)(q13)). COLO232, EJ, KK47, and VM-CUB-III cell lines all had rearrangements affecting chromosome 3p.

Discussion

This study is the first to describe the expression and localization of individual uroplakin gene transcripts in

Table 3. Expression of Uroplakin Transcripts by Normal and Tumor-Derived Urothelial Cell Lines by Ribonuclease Protection Assay

Cell line	UPIa	UPIb	UPII	UPIII
NHU*	- (4/5) + (1/5)	+++ (6/6)	- (7/8) + (1/8)	- (4/4)
RT4	++	+++	+++	++
RT112	-	+++	-	-
HT1376	-	+++	-	-
Colo232	-	-	-	-
KK47	-	-	-	-
VM-CUB-3	++	+++	++	-
Hu456	-	-	-	-
EJ	-	-	-	-

On TCC cells, the hybridization signal was scored as negative (-), weak (+), moderate (++), or strong (+++).

*Numbers indicate cell lines from independent donors.

normal and neoplastic human urothelium. The use of primers designed to consensus sequences from other species demonstrates the high interspecies conservation of the four uroplakin genes.⁴ Nevertheless, UPIa and UPIb, which both belong to the tetraspan family of proteins, were different enough at the nucleotide level to show no significant homology and would have been unlikely to cross-hybridize.

The absent or very low expression of UPIa, UPII, and UPIII in NHU cells *in vitro* is consistent with our previous immunocytochemical characterization of the culture system, which showed that NHU cells in monoculture adopted a highly proliferative basal/intermediate cell phenotype and did not express antigens associated with late/terminal urothelial cytodifferentiation.¹⁰ The constitutive expression of UPIb transcripts by NHU cells was more surprising and, in the light of other data, raises questions over the differentiation-restricted regulation of UPIb gene expression. Our *in situ* hybridization data are consistent with results from a previous immunohistochemical study that used an antiserum recognizing both UPIb and UPIII epitopes⁹ and suggests that UPIb is the least differentiation-restricted of the uroplakins, being detected in upper intermediate and superficial cells. The *in situ* localization of UPIa and UPII to superficial cells in normal human urothelium is also consistent with the immunolocalization patterns obtained using monospecific antisera.⁸ Taken together, these data indicate that expression of the uroplakin genes is regulated at the transcriptional level and that UPIb is expressed at an earlier stage in the differentiation process than the other uroplakins. Conversely, the low level of UPIII mRNA detected by RPA in urothelium, coupled with the lack of expression of this gene in NHU and TCC cell lines, indicates that UPIII exhibits low-level expression restricted to urothelial cells that have achieved terminal differentiation. Such low-level expression would explain the inability to detect UPIII transcripts by *in situ* hybridization. It is notable that UPIII has been detected immunohistochemically in both normal and neoplastic urothelium.⁸ The discrepancy in detection of mRNA and protein probably reflects differences in sensitivity of the techniques and also the intense

and localized nature of the antigen and may indicate that the UPIII protein is relatively stable.

The expression of uroplakin mRNA transcripts in TCC specimens and the TCC-derived cell lines was found to correlate with the degree of cytodifferentiation. Established TCC cell lines have been used as models for urothelial and TCC cell behavior, as they show a range of phenotypes reflecting the urothelial origin and the histological grade of tumor from which they were derived.¹³⁻¹⁶ We have recently shown that TCC cell lines seeded onto a normal urothelial stroma show differentiation and invasive characteristics consistent with different patterns of TCC behavior seen *in vivo*.²¹ The TCC cell lines have been previously ranked in terms of their differentiated phenotype according to the histological grade of the originating tumor and expression of a panel of antigens *in vitro*.^{13,14} The only cell line in which uroplakin expression did not correlate with this ranking was the VM-CUB-3 cell line, which expressed three of the uroplakins and by this criterion had attained a more differentiated phenotype. The RT4 cell line, derived from a grade 1 TCC and capable of forming a well differentiated stratified urothelium when seeded onto a stroma,²¹ was found to constitutively express transcripts for all four uroplakin genes, including UPIII. This suggests that the TCC cell lines, and in particular RT4, will be invaluable as models for urothelial cytodifferentiation and for studying differential regulation and expression of the uroplakin genes.

In accordance with the expression of UPIb by non-terminally differentiated normal urothelial cells *in vivo* and *in vitro*, high transcription of UPIb was found in TCC cell lines of a moderately differentiated phenotype (RT112, HT1376, and VM-CUB-3). The up-regulated expression of UPIb in cell lines was in accord with the overexpression of UPIb seen in the majority of non-invasive papillary TCC specimens investigated here. It also agrees with the immunohistological findings of Wu and colleagues⁹ who showed that in addition to reacting with superficial cells, their cross-reactive UPIII/UPIb antiserum localized to the cytoplasm and borders of many cell layers within papillary bladder carcinomas. The overexpression and derestriction of a protein that is normally strictly differentiation associated in normal urothelium has also been reported for CK20, which is expressed by superficial cells in normal urothelium but shows full-thickness expression in a proportion of non-invasive papillary TCCs.¹² In the case of CK20, retention of a normal differentiation-restricted expression pattern has been linked to tumor nonrecurrence.^{12,22} Any prognostic significance of UPIb expression changes has yet to be determined. However, it appears that CK20 and UPIb could show discoordinate loss, suggesting that the mechanisms and hence significance underlying the dysregulation of each moiety might be different.

UPIb appears to be a good candidate marker of metastatic TCC by virtue of being retained by both primary and secondary tumors. Nevertheless, additional studies in human tissues will be required to determine whether UPIb can be expressed ectopically by non-urothelial tissues, particularly after neoplastic transformation. Although UPIb is restricted to urothelium in bovine tis-

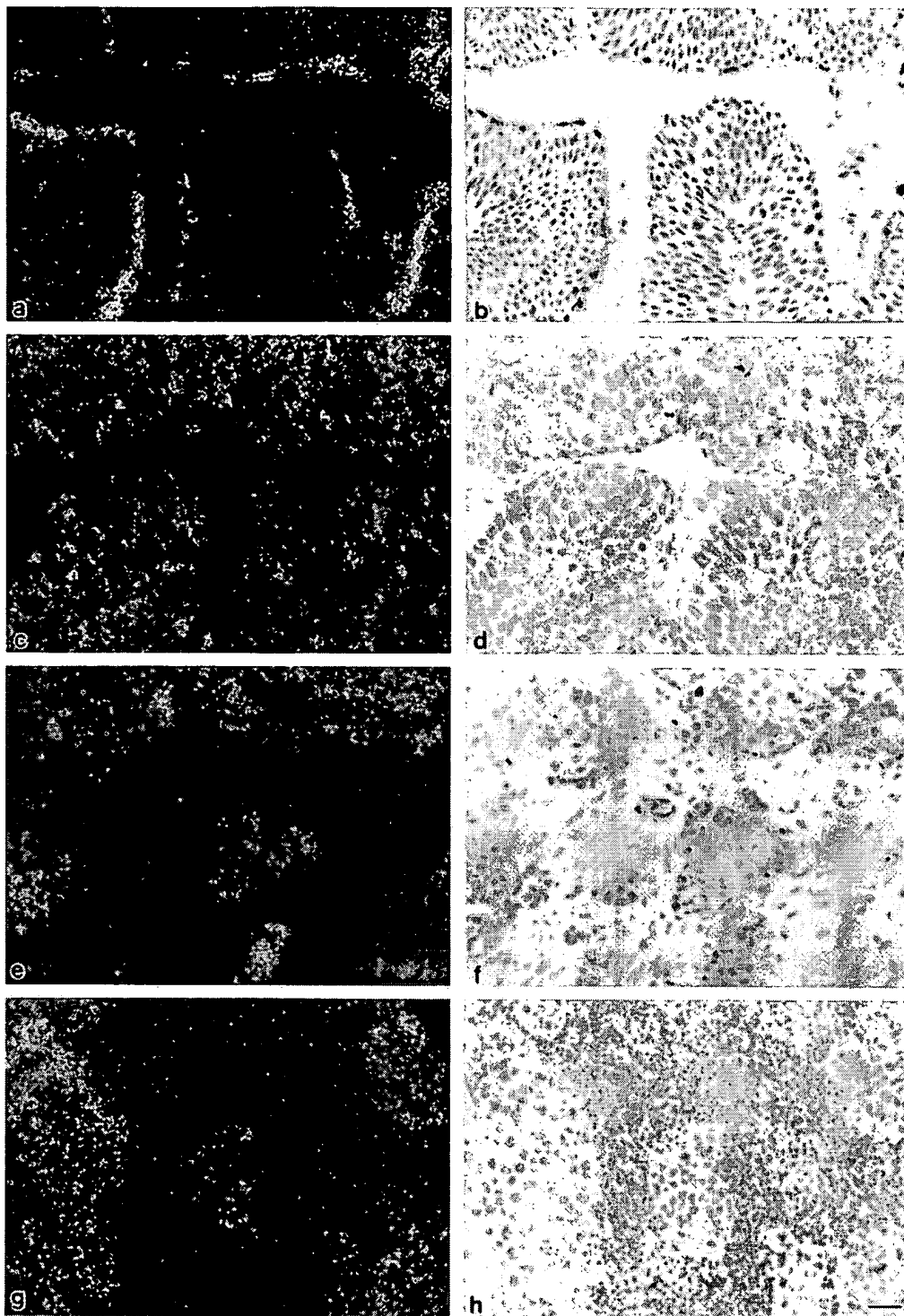


Figure 4. *In situ* hybridization on sections of human TCC using ^{35}S -labeled riboprobes detected by autoradiography and visualized by epi-polarized illumination (a, c, e, and g) or transmitted light (b, d, f, and h). Riboprobes specific for UPIa (a and b) and UPIb (c and d) on well differentiated superficial TCC and UPIb on an invasive TCC (e and f) and a lymph node metastasis (g and h) from the same patient were used. UPIa transcripts were differentiation restricted in TCC and localized to the luminal cells of well differentiated superficial TCC. The expression of UPIb became dissociated from differentiation in neoplastic urothelium, showing extensive expression throughout the tumor in ~50% of noninvasive and invasive tumors and retained in metastatic lesions. Scale bar, 50 μm .

sues,^{3,4} it has been identified as a transforming growth factor- β -regulated gene in mink lung epithelial cells.²³ If the tissue specificity of UPIb is confirmed in human tissues, it could be a useful marker in diagnostic urological

pathology and may complement other urothelial differentiation markers, such as CK20.

The significance of UPIb loss or overexpression in TCC is unclear, both in biological and clinical terms. However,

taken with the expression by nondifferentiated NHU cells *in vitro*, it suggests that UPIb may have more than a structural role in urothelial cell regulation. UPIa and UPIb are both members of the tetraspan family, members of which have been implicated in diverse cellular processes, including cell activation, proliferation, differentiation, adhesion, and motility by facilitating specific interactions between cell surface proteins.²⁴ Several members of the family, including CD63 and CD82, have been implicated as potential metastasis suppressor genes.^{25,26} CD63 has been shown to increase melanoma cell adhesion to extracellular matrices bound via the $\beta 1$ integrins.²⁷ The mapping of UPIb to chromosome 3q13.3-q21,²⁸ which is a region reported as lost in a number of bladder cancers,²⁹⁻³¹ led Finch and colleagues²⁸ to hypothesize that UPIb expression might be altered in bladder cancer. However, the retention of UPIb expression in 50% of lymph node metastases suggests that it is unlikely to function as a metastasis-suppressor gene. In addition, the demonstration that one of the two cell lines with a rearrangement in the critical region of chromosome 3q had retained UPIb expression further suggests that UPIb is unlikely to be the target gene in this region.

The data presented here indicate that expression of UPIa and UPII genes remain highly differentiation restricted in superficial TCC, being expressed by the luminal cells of well differentiated papillae but lost from histologically less differentiated areas. Given the direct relationship in TCC between tumor aggressiveness and histological grade,¹¹ it might be expected that loss of differentiation-associated gene expression might accompany malignant progression. However, the observation of UPII and UPIII antigen expression in over 50% of invasive and metastatic TCCs^{8,9} suggests that even after acquiring a malignant phenotype, neoplastic urothelial cells can undergo an advanced stage of urothelial cytodifferentiation.^{8,9} It has been proposed that urothelial cells can follow a urothelial, squamous, or glandular type of differentiation program and that this competency is reflected in the development of carcinomas of transitional, squamous, and adenocarcinoma types in response to different etiological factors.⁹ The uroplakins have been identified as markers of the normal, or default, urothelial program of differentiation.⁹ However, our data suggest that UPIb may not be strictly differentiation restricted and may have some additional function in urothelial cell or tissue regulation. We have suggested that the highly proliferative and migratory cell phenotype adopted by NHU cells in monoculture is characteristic of a wound response phenotype.³² We hypothesize that expression of UPIb by NHU cells may be part of this response. Investigation of UPIb expression in benign conditions and in urothelial wound-healing models³³ should resolve this issue.

Acknowledgments

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